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Differential effects of TGF-β and FGF-2 on *in vitro* proliferation and migration of primate retinal endothelial and Müller cells

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ABSTRACT.

Purpose: **During retinal development, the pattern of blood vessel formation depends upon the combined effects of proliferation and migration of endothelial cells, astrocytes and Müller cells. In this study, we investigated the potential for transforming growth factor-β (TGF-β) and fibroblast growth factor (FGF-2) to influence this process by regulating proliferation and migration of retinal endothelial and macroglial cells.**

Methods: **We assessed the effects of exogenous TGF-β and FGF-2 on the proliferation and migration of cultured endothelial (RF/6A) and Müller cell (MIO-M1) lines. Cell proliferation was measured using a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay over 72 hr. Cell migration was measured using a scratch-wound assay over 72 hr.**

Results: **Transforming growth factor-β inhibited the proliferation of endothelial and Müller cells and inhibited the migration of Müller cells, but not endothelial cells, compared to untreated controls. Conversely, FGF-2 increased endothelial cell proliferation but inhibited endothelial cell migration. Fibroblast growth factor-2 increased migration of Müller cells but had little effect on proliferation except at higher concentrations (20 ng/ml).**

Conclusion: **Taken together, these observations indicate that TGF-β and FGF could work in concert to inhibit endothelial cell proliferation and migration, respectively; this may have implications for establishing and maintaining the avascular zone of primate fovea.**

Key words: endothelial cells – fibroblast growth factor – foveal avascular zone – Müller cells – primate retina – transforming growth factor-β

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Introduction

A distinguishing feature of the primate retina is the specialized *macula lutea,* which includes the *fovea centralis*. The fovea is a shallow depression in the central retina adapted for high acuity vision. Foveal adaptations include an absence of retinal vessels and rod photoreceptors and centrifugal displacement of inner retinal layers onto the foveal rim (Provis & Hendrickson 2008). Factors that induce specialization of the macula and formation of the fovea remain poorly understood. However, establishment of the foveal avascular zone (FAZ) is a likely key step in delineation of the fovea during development (Provis & Hendrickson 2008). This notion is supported by clinical investigations using optical coherence tomography showing that when the FAZ is absent, the foveal depression is also absent (McGuire et al. 2003).

Vascularization of the retina involves proliferation and migration of astrocytes and endothelial cells, so that the vessels spread outward from the disc and reach the peripheral retina at around birth. Retinal vessels

and their associated astrocytes enter the retina from the optic disc at about 14 weeks' gestation (WG) in humans (Provis & Hendrickson 2008). However, the foveal region is never vascularized during normal development, and the FAZ is fully demarcated by around 28 WG in humans (Provis & Hendrickson 2008). Astrocytes leading the endothelial cell migration into the retina express vascular endothelial growth factor (VEGF) in response to local hypoxia (Sandercoe et al. 2003). Despite the high levels of VEGF expression throughout the developing macula, vessels growing towards the macula advance more slowly than in other parts of the retina (Engerman 1976; Provis et al. 2000). A lower rate of cell proliferation is found in vessels approaching the macula compared to vessels growing towards the periphery in primate fetal retina (Cornish et al. 2005). These observations lead to the hypothesis that expression of a gradient of antiangiogenic or antiproliferative factors such as transforming growth factor-β (TGF-β) or fibroblast growth factor-2 (FGF-2), centred on the incipient fovea, may explain the delayed vascularization of the central retina and establishment of the FAZ. Transforming growth factor-β is strongly expressed in the retina (Anderson et al. 1995) and has previously been shown to inhibit the proliferation of astrocytes (Hunter et al. 1993). In this study, we used *in vitro* primate endothelial and Müller cell models to further investigate the potential for TGF-β and FGF-2 to regulate cell proliferation and migration.

Materials and Methods

Endothelial and Müller cell cultures

Cell lines

A rhesus macaque choroid-retina endothelial cell line (RF/6A, CRL-1780, American Type Culture Collection) and an immortalized human Müller cell line, MIO-M1 [Müller 1 Moorefields Institute of Ophthalmology; Dr A. Limb, Institute of Ophthalmology, University College London (Limb et al. 2002)] were used. Cells were grown in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 units/ml penicillin and 50 units/ml streptomycin and 2mM L-glutamine and maintained in a humidified incubator at 37°C with 5% CO_2. The medium was changed every 3 days, and cells were passaged using 0.25% trypsin/0.1% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) when cells were approximately 80% confluent. All tissue culture reagents were obtained from ThermoTrace Pty Ltd (Australia) unless otherwise specified.

We initially confirmed that MIO-M1 Müller and RF/6A endothelial cell lines expressed receptors for TGF-β and FGF-2, required for cells to respond to the growth factors used in the proliferation and migration assays (not shown).

Proliferation assays

We measured the dose response of TGF-β and FGF-2 on cell proliferation for both cell lines. Endothelial and Müller cells were seeded at 2×10^4 cell/ml in 96-well plates and grown for 24 hr in DMEM growth medium, followed by 24 hr in serum-free medium (SFM). Cells were treated with TGF-β or FGF-2 at concentrations of 4, 8, 12, 16 or 20 ng/ml in SFM ($N = 6$ wells/concentration). Control cells grown in SFM only were also included ($N = 6$ wells). The experiment was repeated on three separate occasions ($N = 18$ wells total). For the proliferation assay, cells were grown for 72 hr then 10 μl of MTT reagent was added to each well and incubated in the dark at 37°C for 4 hr, with 100 μl of solubilization reagent added to the wells overnight to dissolve the formazan crystals. Absorbance was measured at 570 nm with a 650-nm reference (BioRad Microplate Reader, Model 550, Bio-Rad Laboratories, Hercules, CA, USA). Absorbances for untreated control cells were averaged for each separate experiment (C_{avg}). For each growth factor concentration tested, e.g. 2 ng/ml, the absorbance (D) was expressed as a percentage of C_{avg}; that is, $(D/C_{avg}) \times 100\% = \%\ C_{avg}$ Dose–response curves were then plotted for each cell line (% $C_{avg} \pm$ SEM, $N = 18$ for control and each growth factor concentration).

Cell migration assays

We measured the effect of TGF-β and FGF-2 on the migration of Müller and endothelial cell lines using a scratch-wound assay. Cells were seeded in 12-well plates (Müller cells 1×10^5 cells/ml; endothelial cells 7.5×10^4 cells/ml) and grown for 48 hr to ~85% confluence in DMEM, followed a 24-hr adaptation in SFM. Cell proliferation was blocked with 10 μg/ml mitomycin-C for 2 hr. Uniform wounds were then made in confluent cell monolayers using a 1-ml pipette tip drawn vertically across the centre of the well; all wounds were made by an independent investigator (MCM). This technique produced a consistent wound devoid of cells, ~5 mm long × 600 μm wide. Wells were then gently washed twice with PBS to remove any cell debris, and the wounds were assessed for consistency.

Cells were treated with either TGF-β (10 ng/ml), FGF-2 (10 ng/ml) or, for controls, SFM. The growth factor concentration used was determined empirically from dose–response assays. For each experiment, four wells per treatment group were assessed ($N = 4$ per growth factor). Digital images of wound areas were captured using a 4× objective on a phase-contrast light microscope Olympus IX71 with a DP70 camera (Olympus, Tokyo, Japan) and DP Controller v1.2.1.107 software (16 bits/channel, 1024 × 1024 pixels), and wound areas were viewed at $T = 0$, 6, 12, 24 and 48 hr postscratch. The experiment was repeated twice on separate occasions, giving a total of $N = 8$ wells for analysis for each condition. At the completion of all experiments, wound areas were analysed using the FoveaPro Plug-in (Reindeer Graphics) for Adobe PhotoshopCS2. The wound edge was traced by hand, and the software was used to select and calculate the area of the wound. The area is calculated by determining the number of pixels within the traced area and scaling to actual dimensions.

The order of growth factor administration, cell type and wounding within the 12-well plates was randomized for each experiment to minimize bias. All wound preparations were maintained under identical conditions before analysis. The distinct morphology of each cell type made masking

of all experimental conditions unfeasible. To ensure consistency of measurements and reduce bias, a separate investigator (MCM) produced the scratch-wounds with the same sized pipette tip; however, the precise size of the measured wound varied (reported in results). All images were collected and analysed by one investigator (PR), and the same wound location was imaged at each time-point, for each experimental condition. Record of the initial wound size and analysis of the final wound size were unknown to the investigator tracing the area until objective calculation of pixel dimensions by the software.

Statistical analysis

Wound areas were calculated as per cent coverage compared to the initial wound area ($T = 0$) to account for any variation between initial wound areas. Nonlinear regression analysis was performed in SIGMAPLOT v9.0 (Systat Software, Chicago, IL, USA) for all values at respective time-points. The rate of migration was modelled against an increase to maximum, 2-parameter curve, and the equation of curve was determined. Statistical differences in the response to TGF-β and FGF-2 were determined by polynomial curve fitting:

$$F = \frac{(\text{regression of SS for higher model}) - (\text{regression SS for lower degree model})}{\text{residual MS for higher model}}$$

Critical values of the F distribution were taken as F(critical) [$\alpha(1)$; p = 0.01; df = 36] = 7.31.

Results

Fibroblast growth factor-2-treated endothelial cells showed a dose-related increase in proliferation to 116% of controls (2–20 ng/ml FGF-2; Fig. 1A). For most concentrations of FGF-2 (2–16 ng/ml), Müller cell proliferation did not differ from untreated cells; however at 20 ng/ml, cell growth was reduced to 90% of control levels (Fig. 1A). Transforming growth factor-β (2 ng/ml) significantly decreased proliferation relative to medium alone, for both endothelial (85%) and Müller cells (87%) (Fig. 1B).

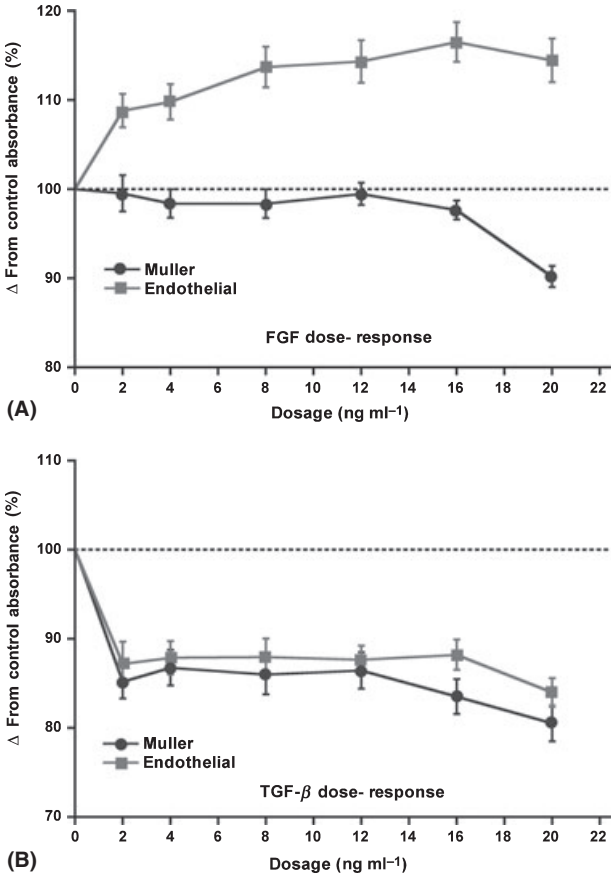

Fig. 1. Cell proliferation *in vitro*. (A) transforming growth factor-β treatment decreased endothelial and Müller cell proliferation at doses from 2 to 20 ng/ml (B) fibroblast growth factor (FGF-2) treatment increased endothelial cell proliferation at all doses. Conversely, Müller cell proliferation remained unchanged with 2–16 ng/ml; a 10% decrease in proliferation from control levels is seen with 20 ng/ml FGF-2.

Cell migration *in vitro* was measured using a scratch-wound assay, with different patterns of wound healing observed for each cell type and growth factor (Fig. 2). During endothelial cell migration, the cells at the wound front lost their characteristic 'cobblestone' arrangement and appeared to extend filopodia into the wound area. Transforming growth factor-β -treated Müller cells extended long thin processes into the wound area, while cell somata remained at the wound front. In contrast, FGF-2-treated Müller cells displayed rapid migration into the wound area. These migrating cells extended multiple short thickened cell processes, and individual cells were observed within the wound area. In contrast to the TGF-β -treated cells, the cell bodies did not remain at the wound front (Fig. 2).

Endothelial cells and Müller cells displayed distinct cell migration rates over 48 hr, in response to TGF-β and FGF-2 (Table 1). In control SFM, endothelial and Müller cells showed different migration rates. Endothelial cell migration was significantly more rapid compared to Müller cells, and at 48 hr, there was 45% and 55% wound area coverage for Müller and endothelial cells, respectively ($p < 0.01$) (Fig. 3). Müller cell migration was significantly inhibited by TGF-β (10 ng/ml) to almost half the migration of controls ($p < 0.01$) (Fig. 3). In contrast, FGF-2 (10 ng/ml) significantly promoted Müller cell migration consistently across all time-points ($p < 0.01$), reaching 100% wound coverage by 48 hr. The response of endothelial cells to TGF-β and FGF-2 was very different compared to Müller cells. Endothelial cells in TGF-β showed a similar migration pattern to

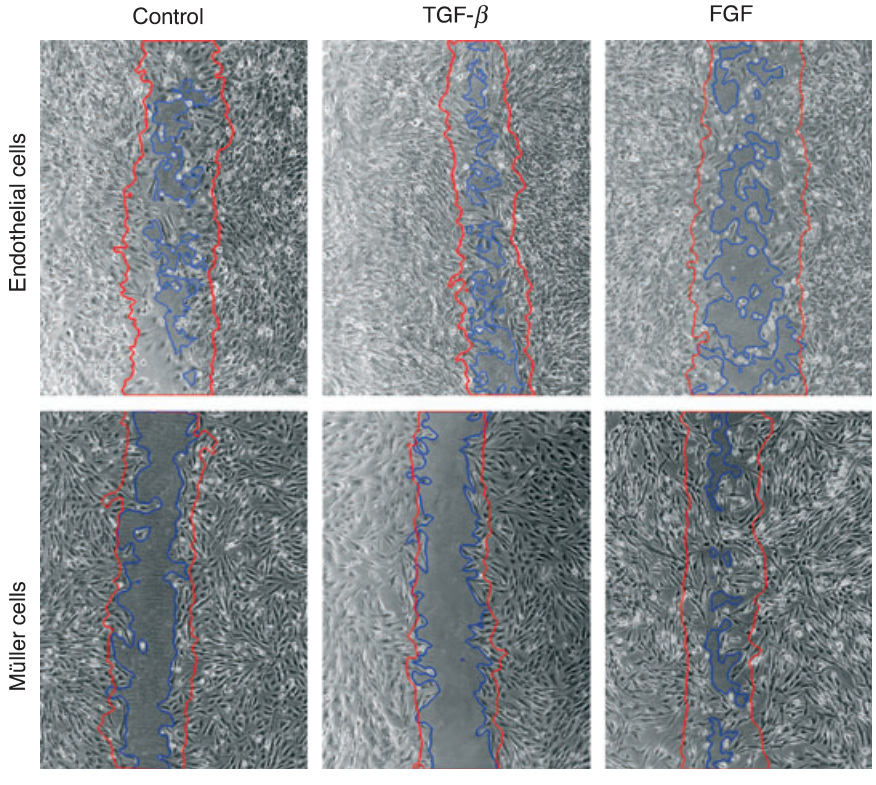

Fig. 2. Cell migration assays. Wound areas at $T = 48$ hr (blue outline) compared to areas at $T = 0$ (red outline) for Müller and endothelial cells. These representative images show the different patterns of cell migration for each cell type.

Table 1. Nonlinear regression analysis of migration rates.

Migration rate comparison	*F*	*F*(critical)	p value
Control Müller versus control endothelium	10.89	7.31	$p < 0.01$*
Endothelium TGF-β versus control	6.75	7.31	$p > 0.01$
Endothelium FGF-2 versus control	17.77	7.31	$p < 0.01$*
Müller cell TGF-β versus control	22.77	7.31	$p < 0.01$*
Müller FGF-2 versus control	22.82	7.31	$p < 0.01$*

Comparison of migration rates in transforming growth factor-β (TGF-β) and fibroblast growth factor (FGF)-2 treated and control (untreated) Müller and endothelial cells. Significant differences in migration rates occurred for all comparisons except for TGF-β -treated endothelial cells. $N = 38$; *F*(critical): [$\alpha(1)$; $p = 0.01$; $df = 36$] = 7.31. * Significant differences between the two conditions; $p < 0.01$.

controls ($p < 0.01$), with a slight increase in migration at 48 hr. In contrast, endothelial cells grown in FGF-2 demonstrated a significant inhibition of migration over 48 hr, achieving only 36% wound coverage compared to 55% coverage in controls ($p < 0.01$) (Fig. 3).

Discussion

In this study, TGF-β inhibited the proliferation of endothelial and Müller cells and inhibited the migration of Müller cells, but not endothelial cells, compared to untreated controls. Conversely, FGF-2 increased endothelial cell proliferation but inhibited endothelial cell migration and increased migration of Müller cells but showed minimal effects on Müller cell proliferation except at higher concentrations (20 ng/ml). These findings suggest that the combined effects of TGF-β and FGF-2 could contribute to the differential growth responses of endothelial cells and Müller cells observed during normal retinal development and in pathology.

Demarcation of the incipient fovea is a perplexing issue. At no time during development is the incipient or developing fovea vascularized (Provis & Hendrickson 2008). As vessels approach the incipient fovea, their rate of growth decreases, and endothelial cell proliferation is decreased in the vicinity of the incipient fovea and along the horizontal meridian, compared to the rest of the retina (Sandercoe et al. 2003). It has been suggested that antiproliferative and/or antiangiogenic factor(s) are differentially expressed along the horizontal meridian and concentrated at the incipient fovea. As well as controlling proliferation, these factors could limit migration of key cell types to produce a complex morphological pattern consistent with that observed during foveal development. Consequently, the presence of such factor(s) would play an important role in preventing vessel growth into the central fovea (Provis 2001). The expression of such factor/s would need to occur prior to any physical marking of the FAZ by the vasculature and associated cell types. Indeed, it has been reported recently that the antiangiogenic factors, PEDF and brain natriuretic peptide, along with the guidance factor EphA6, are highly expressed in the developing foveal region (Kozulin et al. 2009a,b).

Several growth factors including VEGF, TGF-β and FGF-2 are involved in the highly complex and well-controlled processes of primate retinal vascular development (Sandercoe et al.2003; Cornish et al. 2005). Consistent with these observations, our *in vitro* study shows that both TGF-β and FGF-2 can inhibit endothelial cell proliferation and migration, respectively, suggesting the interaction of these growth factors may play an important role in maintaining an avascular incipient fovea during primate retinal development.

The role of FGF-2 in eye development has primarily related to its role in lens development, where FGF-2 is critical in regulating the differentiation of lens epithelial cells to elongated fibre cells in a dose-dependent manner -reviewed in (Lovicu & McAvoy 2005). A definitive role for FGF-2 in retinal vascular growth has not yet been established. A normal retinal vasculature was found to develop in FGF-2 knockout mice; however, this most likely is because of a redundancy in growth factors and receptors

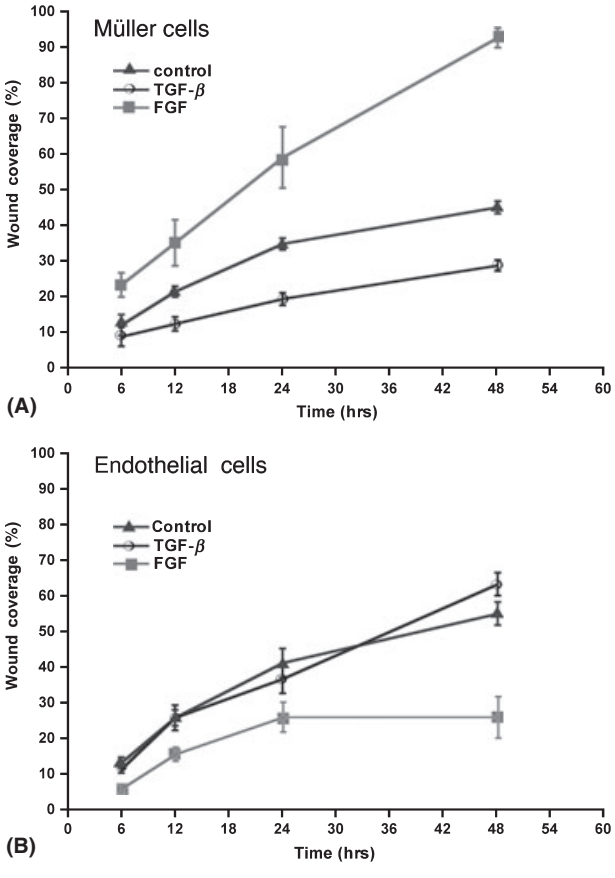

Fig. 3. Migration of (A) Müller cells and (B) endothelial cells. For Müller cells, a significant increase in migration is induced by transforming growth factor-β (TGF-β) (10 ng/ml) compared to control serum-free medium ($p < 0.01$) and a significant decrease is seen with fibroblast growth factor (FGF-2) (10 ng/ml) ($p < 0.01$). For endothelial cells, the migration rates are similar for TGF-β-treated and control (serum-free) cells, compared to a significant decrease in migration induced by FGF-2 ($p < 0.01$).

(Ozaki et al. 1998). *In vitro* studies have suggested that FGF-2 stimulates the expression of VEGF by endothelial cells (Pepper & Mandriota 1998). Fibroblast growth factor-2 has also been reported to play a major role in the development of cones in the foveal region in primate retina, with the establishment of an FGF-2 mRNA gradient leading to localized elongation and thinning of foveal cones (Cornish et al. 2005). In the current study, we showed that FGF-2 has a differential effect on the proliferation and migration of Müller cells and endothelial cells. Fibroblast growth factor-2 increased endothelial cell proliferation with no effect on Müller cell proliferation. In contrast, FGF-2 promoted migration of Müller cells *but* inhibited endothelial cell migration. This finding is significant, as it shows that a complex pattern of advancement, expansion and suppression of growth of two key retinal cell types can potentially be affected by combinations of growth factors that display gradients of expression in the developing primate retina.

Transforming growth factor-β is implicated in regulating the differentiation, proliferation, migration and survival of many cell types. The three isoforms: TGF-β1, TGF-β2, TGF-β3 overlap in their respective functions (Lebrin et al. 2005). Transforming growth factor-β2 can act on either of two transmembrane serine/threonine kinase receptors, TβR-II and two TβR-I, one of which is restricted to the endothelium (Lebrin et al. 2005).

In vitro, TGF-β can either increase or decrease endothelial cell proliferation, dependent on the concentration, where low doses of TGF-β stimulate and high doses inhibit endothelial cell proliferation (Goumans et al. 2002). This balance is thought to be regulated by ALK5 (which leads to the inhibition of migration and proliferation) and ALK1 (which induces migration and proliferation) signalling (Goumans et al. 2002). Abnormalities arising from gene knock-out of TGF-β receptors and ligands (Larsson et al. 2001; Dickson et al. 1995) have demonstrated the significant role that TGF-β signalling plays in vascular development. Transforming growth factor-β has been shown to indirectly inhibit endothelial cell proliferation and the expression of VEGF receptor II in these cells (Lebrin et al. 2005).

Developmentally, Müller cells, photoreceptors and a subset of inner retinal neurons descend from a single retinal progenitor cell (Turner & Cepko 1987). Müller cells are derived from the end-stage differentiation of these progenitor cells (Furukawa et al. 2000) and provide a template for vascular growth and patterning (Holländer et al. 1991). In the developing retina, Müller cells are involved in the phagocytosis of apoptotic neurons or pigment epithelial cells (Egensperger et al. 1996). In the mature retina, Müller cells play an important role in stabilizing the architecture of the retina by providing structural support for both vasculature and neuronal components (Zhang & Stone 1997). The promotion of Müller cell process extension and migration by FGF-2 and TGF-β observed here has implications for the Müller cell hypertrophy and invasion commonly seen in retinal diseases, including age-related macular degeneration.

We have demonstrated that TGF-β can inhibit the proliferation of endothelial cells and Müller cells. In response to hypoxia, Müller cells release TGF-β that can inhibit proliferation of retinal neurons and Müller cells, in a paracrine and autocrine fashion, respectively (Yafai et al. 2004). Transforming growth factor-β, along with other cytokines, has been implicated along with other cytokines in the regulation of matrix metalloproteinases, which play a crucial role in normal physiological, developmental and pathological processes of angiogenesis and wound healing in the eye (Eichler et al. 2002). In this study, the rate of Müller cell proliferation decreased with TGF-β, similar to the response observed for endothelial cells. Taken together, the present observations suggest that TGF-β and FGF-2 may be involved in defining the FAZ by differential regulation of

endothelial and Müller cell proliferation and migration.

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